Influence of carbohydrates on lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*

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KEYWORDS: Cellulaseless mutant*, Sporotrichum pulverulentum*, Cellobiose: quinone oxidoreductase*, Cellulase, Laccase, Lignins, Carbohydrates.

SUMMARY: Production of biological pulp implies selective removal of lignin from wood chips with the aid of microorganisms. The enzyme cellobiose:quinone oxidoreductase, which seems to be of importance for lignin degradation, also requires, however, degradation of cellulose. This paper presents an investigation into the question of whether a white-rot fungus could be genetically manipulated so as to make it unable to degrade cellulose while still retaining the ability of degrading lignin.

The white-rot fungus *Sporotrichum pulverulentum* and a cellulase-less mutant strain (Cel 44) have been cultivated on agar plates containing kraft lignin and different carbohydrates, and also on birch, pine and spruce splintwood.

Cel 44 could degrade both kraft lignin and lignin in wood without having cellulase or cellobiose:quinone oxidoreductase activity. The wild type *S. pulverulentum* which has both of these enzyme activities, however, degraded kraft lignin better than the mutant. The presence of cellobiose:quinone oxidoreductase is not entirely necessary since in 10 weeks Cel 44 degraded 31% of the lignin without loss of cellulose, 32% of xylan being degraded at the same time.

From the extent of coloration in the kraft lignin agar plates, it was deduced that the production of both laccase and cellobiose:quinone oxidoreductase is repressed by easily metabolized carbohydrates. When high DP carbohydrates were present, no repression occurred and the lignin was strongly degraded by the wild type. Cel 44 degrades lignin almost independently of the DP of the carbohydrate.

□ Framställning av biologisk massa innebär att lignin selektivt löses ut ur vedflis med hjälp av mikroorganismer. Enzymet cellobios:kinon oxidoreduktas, som tycks vara viktigt för ligninnedbrytningen, fordrar emellertid också nedbrytning av cellulosa. I detta arbete presenteras en undersökning som besvarar frågan, huruvida en genetiskt manipulerad vitrötasvamp, som inte längre kan bryta ned cellulosa, ändå kan bryta ned lignin.

Vitrötasvampen *Sporotrichum pulverulentum* och en cellulasnegativ mutant (Cel 44) har odlats på agarplattor innehållande kraftlignin och några olika kolhydrater samt på splintved av björk, tall och gran.

Cel 44 kan bryta ned både kraftlignin och lignin i ved utan att ha vare sig cellulas- eller cellobios:kinon oxidoreduktasaktivitet. Vildtypen av *S. pulverulentum*, som har båda enzymaktiviteterna, kunde emellertid bryta ned kraftlignin bättre än mutanten. Cellobios:kinon oxidoreduktas är emellertid inte helt nödvändigt då Cel 44 på 10 veckor bröt ned 31 % av ligninet i björkved utan cellulosaförlust. Xylanmängden reducerades dock med 32 %.

Graden av färgning i kraftligninplattorna visade att produktionen av både lackas och cellobios:kinon oxidoreduktas hämmas av lätt metaboliserbara kolhydrater. Om kolhydrater med högt DP finns närvarande erhålls ingen repression av dessa enzymer och ligninet bryts ned kraftigt av vildtypen. Cel 44 bryter ned lignin nästan oberoende av kolhydratens DP-värde.

□ Die Herstellung von „biologischem Zellstoff" beruht darauf, dass Lignin mit Hilfe von Mikroorganismen selektiv aus Hackschnitzeln herausgelöst wird. Das Enzym Cellobiose:Chinon Oxydoreduktase, welches für den Ligninabbau wichtig zu sein scheint, erfordert jedoch auch den Abbau von Cellulose. In der vorliegenden Arbeit wird eine Untersuchung beschrieben, die die Frage beantwortet, inwiefern ein genetisch veränderter Weissfäulepilz, der Cellulose nicht mehr abbauen kann, dennoch zum Ligninabbau befähigt ist.

Der Weissfäulepilz *Sporotrichum pulverulentum* und eine Cellulase-negative Mutante (Cel 44) wurden auf Agarplatten, welche Sulfatlignin und einige verschiedene Kohlenhydrate enthielten, sowie auf Splintholz von Birke, Kiefer und Fichte, gezüchtet.

Cel 44 konnte sowohl Sulfatlignin, wie auch natives Lignin abbauen, ohne Cellulase- und Cellobiose:Chinon Oxydoreduktaseaktivität zu besitzen. Der Wildstamm von *S. pulverulentum*, welcher beide Enzymaktivitäten besitzt, konnte jedoch Sulfatlignin besser abbauen als die Mutante.

Cellobiose:Chinon Oxydoreduktase ist jedoch nicht unbedingt erforderlich, da Cel 44 innerhalb von 10 Wochen 31 % des Lignins in Birkenholz ohne Celluloseverlust abbaute. Die Menge Xylan nimmt hierbei um 32 % ab.

Die Farbintensität des Sulfatlignins in den Agarplatten zeigte, dass die Produktion sowohl von Laccase als auch von Cellobiose:Chinon Oxydoreduktase durch leicht metabolisierbare Kohlenhydrate gehemmt wird.

In Anwesenheit von hochmolekularen Kohlenhydraten wird keine Zurückdrängung dieser Enzyme beobachtet und das Lignin wird vom Wildstamm stark abgebaut. Cel 44 baut Lignin fast unabhängig vom DP-Wert der Kohlenhydrate ab.

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Wood is decomposed mainly by rot fungi belonging to the Basidiomycetes. One type—the brown-rot fungi—utilizes the carbohydrates and leaves the lignin only slightly affected (1). White-rot fungi, however, metabolize the lignin as well as the carbohydrates (1).

When wood is decayed by a typical white-rot fungus such as, for example, *Polyporus versicolor*, all the main components are removed approximately simultaneously (2, 3). Decayed wood, however, has been found in nature with a very low content of lignin (4). Kirk and Moore (5) reported that the two white-rot fungi *Fomes ulmarius* and *Polyporus berkeleyi* removed lignin faster than cellulose and hemicellulose.

When the ability of different fungi to degrade lignin has been investigated, different lignin preparations have been used, for example, such as Brauns' native lignin, Klason lignin, Björkman lignin (MWL), lignosulphonate or kraft lignin. However, very little has been reported on the degradation of kraft lignin. Gottlieb et al. (6) reported that the two white-rot fungi *Polyporus abietinus* and *Poria subacida* did not grow on kraft lignin in standing liquid cultures. This might have been an effect of the cultivation technique, since Eriksson and Goodell (7) found that the white-rot fungus *Polyporus adustus* and several cellulase-less mutant strains of that fungus could degrade kraft lignin in solid agar media in the presence of glucose. Recently Hiroi and Eriksson (8) have found that MWL, lignosulphonate and kraft lignin are degraded by *Pleurotus ostreatus* regardless of whether cellulose were present or not.

Westermark and Eriksson (9, 10) reported the discovery of a new enzyme called cellobiose:quinone oxidoreductase (the trivial name cellobiose dehydrogenase is used throughout in this report) in *Polyporus versicolor* when the fungus was grown on kraft lignin agar plates supplemented with cellulose or cellobiose. This enzyme reduces quinones or phenoxy radicals formed by laccase during lignin degradation and simultaneously oxidizes cellobiose to cellobiono-δ-lactone. Since the enzyme is also induced by cellulose and cellulose degradation prod-

ucts alone, it was suggested that the enzyme might be involved in degradation of both cellulose and lignin in wood.

The aim of this work has been to find out if wood could be delignified without degradation of cellulose. It was also considered important to investigate whether or not cellobiose dehydrogenase was essential for lignin degradation.

A mutant strain of the white-rot fungus *Sporotrichum pulverulentum* lacking the five endo-1,4-β-glucanases and the exo-1,4-β-glucanase (11) was therefore isolated and cultivated in liquid culture and it was studied whether cellobiose dehydrogenase could be produced. The mutant and the wild type *S. pulverulentum* were also cultivated on kraft lignin agar plates in the presence of different carbohydrates. The induction of laccase and cellobiose dehydrogenase was studied in relation to the degradation of the kraft lignin. The removal of lignin, cellulose, and hemicellulose by the wild type and the mutant from birch, pine, and spruce sapwood was also studied.

Materials and methods

Organism

Sporotrichum pulverulentum Novobranova formerly called *Chrysosporium lignorum*. It was isolated by Dr. Thomas Nilsson, Royal College of Forestry, Stockholm, Sweden. The fungus was first classified as belonging to Fungi imperfecti but is now regarded as a Basidiomycete (11—13).

Solid media

All cultivations in solid media were carried out with the nutrient salts (NS) and the Noble agar described by Eriksson and Goodell (7). The pH-value was adjusted to 5.5 before autoclaving. Walseth cellulose (14) was made from Munktell's Cellulose Powder No. 400 (Grycksbo, Sweden). The resulting DP of the amorphous cellulose was determined viscometrically according to the SCAN standard method (15) and found to be 150. The xylans used were from Wood Gum (Nutritional Biochemicals Corporation, Cleveland, Ohio, USA) or from Larch (Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire, England). Glucomannan from *Pinus silvestris* was obtained from the Pulp Technology Department of this laboratory. It contained 78% mannose, 18% glucose, 1.2% arabinose, 1.4% xylose and 1.4% galactose. Cellobiose and glucose were of pro analysi quality. Cotton linters with DP 2000 and 500 respectively were obtained from Peter Temming AG, Glückstadt, Germany. Indulin AT, a kraft lignin preparation from softwood, was obtained from Westvaco, Polychemical Div., Charleston, S.C., USA. The lignin was washed several times with distilled water to remove low molecular weight compounds and impurities. The washed lignin was dissolved in 0.2 N KOH and sterilized by filtration through a Seitz-filter EKS (Seitz-Werke GmbH, Bad Kreuznach, Germany) before use.

Mutagenesis and selection of cellulase-less mutants

Mutagenesis was carried out according to Eriksson and Goodell (7) with the following modifications: Spores from *S. pulverulentum* were irradiated with UV-light for 15 minutes. With this treatment about 5% of the spores survived. The spores were plated onto petri plates containing 20 ml 1.5% Noble agar and NS and were incubated overnight. Thereafter 8 ml of agar, NS, Walseth cellulose (1.0%) and glucose (0.05%) were layered over the bottom layer. To obtain colonial growth the top layer also contained 0.8% saponin (E. Merck AG).

After two weeks about 20 surviving spores on each plate had formed colonies 5—10 mm in diameter. Colonies which could not produce clear zones in the cellulose agar were further tested in small vials according to (7). One cellulase-less mutant (Cel 44) was found out of 3×10^4 colonies screened on the plates.

Degradation of cellulose, xylan and glucomannan

Wild type (WT) and the cellulase-less mutant (Cel 44) of *S. pulverulentum* were tested for formation of clear zones in Walseth cellulose, xylan and glucomannan according to Rautela and Cowling (16). The tubes contained 1% of one of the above carbohydrates, 0.125% yeast extract and 0.1% glucose plus NS and 1.5% Noble agar. In addition to this Cel 44 was also tested on a modified Bravery-medium according to Nilsson (17). This medium contained 0.25% Walseth cellulose but no yeast extract or glucose.

Enzyme production

All cultivations in submerged culture were performed in a modified Norkrans medium as described by Ahlgren and Eriksson (18). One litre Erlenmeyer-flasks with 300 ml of the medium were shaken on a rotary shaker at 150 rpm with a shaking diameter of 40 mm for 7 days. When endo-1,4-β-glucanase production was investigated the flasks also contained 0.5% Munktell's Cellulose Powder, 0.1% glucose and 0.125% yeast extract (Difco). Production of cellobiose dehydrogenase was tested on a) 0.3% and 1.0% powder cellulose plus 0.1% glucose b) 0.1% and 0.3% cellobiose.

After shaking, the mycelium was filtered off on a sintered glass funnel and the culture solution was centrifuged. Where necessary, the solutions were concentrated in a cold room in collodion tubes (SM 13 200, Sartorius-Membrane filter GmbH, Göttingen, Germany).

Enzyme assays

Endo-1,4-β-glucanase (E.C.3.2.1.4) was determined viscometrically at 25°C and at pH 5.0 as described by Almin and Eriksson (19) and by Ahlgren et al. (20).

One enzyme unit is the amount which catalysis the cleavage of glycosidic bonds at a rate of 1 μmol per minute under the condition defined by the Enzyme Commission.

The activity of cellobiose:quinone oxidoreductase (cellobiose dehydrogenase) was determined according

to Westermark and Eriksson (10) with 3-methoxy-5-*tert*-butyl-benzoquinone-(1, 2) (prepared at this laboratory) and cellobiose as substrates. The reduction of the quinone was followed at 360 nm using a Zeiss spectrophotometer PMQII at 25°C and pH 4.5.

One enzyme unit is the amount of enzyme which reduces 1 μmol of quinone per minute under the conditions defined by the Enzyme Commission.

Qualitative test for phenol oxidases

Laccase was detected in WT and Cel 44 using the drop test method according to Käärik (21). In this method, alcoholic solutions of laccase substrates are dropped onto malt agar plates with actively growing mycelium. Different colour formations in the agar are taken as a positive indication of laccase production.

Degradation of kraft lignin

WT and Cel 44 were grown on double layer agar plates for one month at 25°C and 70% relative humidity. The bottom layer contained 20 ml 1.5% Noble agar plus NS, while the top layer (8 ml) also contained 24 mg lignin plus one of the following carbohydrates: cotton linters DP 2000, cotton linters DP 500, Walseth cellulose DP 150, cellobiose, glucose, xylan (Wood gum) or glucomannan. The amounts of these carbohydrates per plate were 0, 6, 12, 24 or 48 mg, which corresponds to 0%, 0.075%, 0.15%, 0.30% and 0.60% carbohydrate in the top layer.

After incubation, the mycelium on the surface was carefully scraped off the agar. The agar was homogenized in a MSE Homogenizer (Measuring and Scientific Equipment Ltd, London, England) with 100 ml distilled water. The resulting suspension was diluted to 385 ml and the lignin content determined by the chlorine consumption method according to Kyrklund and Strandell (22). A standard curve was prepared from the results for agar plates with known amounts of lignin. The analyses were always carried out with two sets of plates. In one set the top layer contained nutrient salts, agar, lignin and carbohydrate while in the other set the lignin was excluded. The difference between the chlorine numbers of the two sets was taken as a measure of the amount of kraft lignin present in the plates.

It has been shown by Kirk and Lundqvist (23) that white-rotted MWL does not differ significantly from sound MWL. On the other hand, Hiroi and Eriksson (8) have shown that the chlorine number of decayed lignosulphonate is lower than that of sound lignosulphonate. Thus, the chlorine number does not only reflect a decrease in the total amount of lignin but also a decrease in chlorine absorbing units in the lignin molecule during decay. This is a limitation of the chlorine number method that is hard to overcome.

Degradation of wood

Sapwood blocks (20×20×10 mm) of birch *(Betula verrucosa)*, pine *(Pinus silvestris)* or spruce *(Picea abies)* were placed in 12 g (150 ml) air dry vermiculite (Weibull AB, Stockholm, Sweden) in 250 ml Erlenmeyer flasks according to Henningsson (24). Each flask contained three blocks. To test the influence of humidity, flasks containing 20, 30, 40, 50, 60, 70 and 80 ml distilled water were prepared. After autoclaving for 20 minutes the flasks were inoculated with a mycelial suspension of wild type *S. pulverulentum* grown on 1.5% malt agar plates for two weeks. After incubation for four weeks at 25°C and 70% rel. humidity, the blocks were weighed immediately after withdrawing and after drying at 105°C overnight. Weight losses were calculated as the percentage loss of the original dry weight. Moisture content was calculated as the percentage of water compared with the dry weight after incubation. The initial moisture content in the three wood species was measured in separate flasks containing 60, 70 and 80 ml distilled water respectively after incubation for one day without fungus.

To investigate the degradation of wood components, 60 ml of 1% malt extract (Difco) was added to the vermiculite flasks containing the wood blocks. After autoclaving, the flasks were inoculated with WT and Cel 44. Two flasks were withdrawn after 4, 6, 8 and 10 weeks respectively. After 7 weeks 10 ml sterile water was added to the remaining flasks to compensate for loss of water. Moisture content and weight losses were calculated as before. The dried unextracted wood blocks were ground in a Wiley mill to pass a 40 mesh screen. The ground wood was then analysed for its glucan, xylan and mannan content after hydrolysis in 72% sulphuric acid as described by Sjöström et al. (25). Glucose in the hydrolysate was determined with glucose oxidase. All carbohydrates were separated and quantitatively analysed in a Perkin-Elmer 900 gas chromatograph taking the enzymatically determined glucose as a reference.

In birch glucomannan the ratio of glucose to mannose is 1:1, whereas in softwood the ratio galactose:glucose:mannose in galactoglucomannan is 1:1:3 (26). After hydrolysis in sulphuric acid, the glucose in these mannans is released. It was necessary, therefore, to correct the glucan values for the glucose in the mannans in order to obtain an estimate of the cellulose content.

Results

Isolation and characterization of the cellulase-less mutant

Eriksson and Goodell (7) reported the isolation of several cellulase-less mutants from the white-rot fungus *Polyporus adustus*. Their method was also successful in obtaining mutants of the white-rot fungus *S. pulverulentum*. *P. adustus* grows in colonial form at pH 3, while colonial growth of *S. pulverulentum* had to be induced by addition of saponin. Saponins are reported (27, 28) to cause changes in eucaryotic cell membranes and in the salt balance of the cells. The concentration of the saponin used here does not inhibit cellulolytic activity.

The wild type (WT) and the cellulase-less mutant

Cel 44 were tested for degradation of Walseth cellulose, xylan and glucomannan in test tubes as described under Material and methods. The mutant had not formed a detectable clear zone in the cellulose agar after one month. The Bravery medium was also used, since Nilsson (17) showed that greater clear zones were obtained in this medium than in media containing yeast extract. In xylan and glucomannan traces of clear zones were observed after one month. The clear zones produced by the wild type were 14 mm deep for all carbohydrates after 20 days.

Enzyme production by WT and Cel 44

Induction of endo-1,4-β-glucanase and cellobiose dehydrogenase was tested in submerged cultures after 7 days of growth. With Cellulose Powder plus glucose and yeast extract, 4500 endo-1,4-β-glucanase units per litre culture solution were obtained with the wild type. With Cellulose Powder plus glucose or with only cellobiose the WT gave 6 and 0.5 cellobiose dehydrogenase units respectively. In the case of Cel 44, no activity of either endo-glucanases or cellobiose dehydrogenase could be detected in any of these media after concentration 50 times. The growth of Cel 44 was comparable to the growth of WT.

The production of phenol oxidases was tested according to Käärik (21) and it was found that both WT and Cel 44 produce laccase but not tyrosinase. The following substances give positive reactions with both WT and Cel 44: Benzidine, α-naphtol, *o*-anisidine, α-naphtylamine, guaiacol, 8-oxyquinoline, 2,5-xylidine, *o*-toluidine, gallic acid, tannic acid, pyrogallol, *p*-quinone, aniline, phenol.

Degradation of kraft lignin by WT and Cel 44 in the presence of different carbohydrates

The degradation of kraft lignin by both WT and Cel 44 in agar plates also provided with Walseth cellulose was measured by the chlorine number method. *Fig. 1* shows that when cellulose is present WT can degrade 60% of the lignin within three weeks. In the absence of cellulose, only 36% is degraded. In the same period of time Cel 44 degrades only about 20% lignin with cellulose present and 10% in the absence of cellulose. This result indicates that Walseth cellulose favours lignin degradation by both WT and Cel 44.

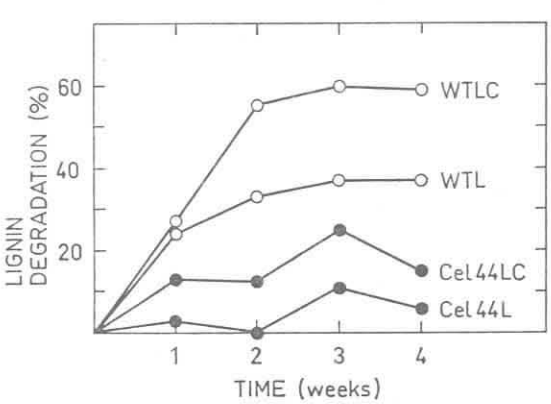

Fig. 1. Degradation of kraft lignin by WT and Cel 44 as a function of time. L=24 mg lignin per plate. LC=24 mg lignin plus 48 mg Walseth cellulose per plate.

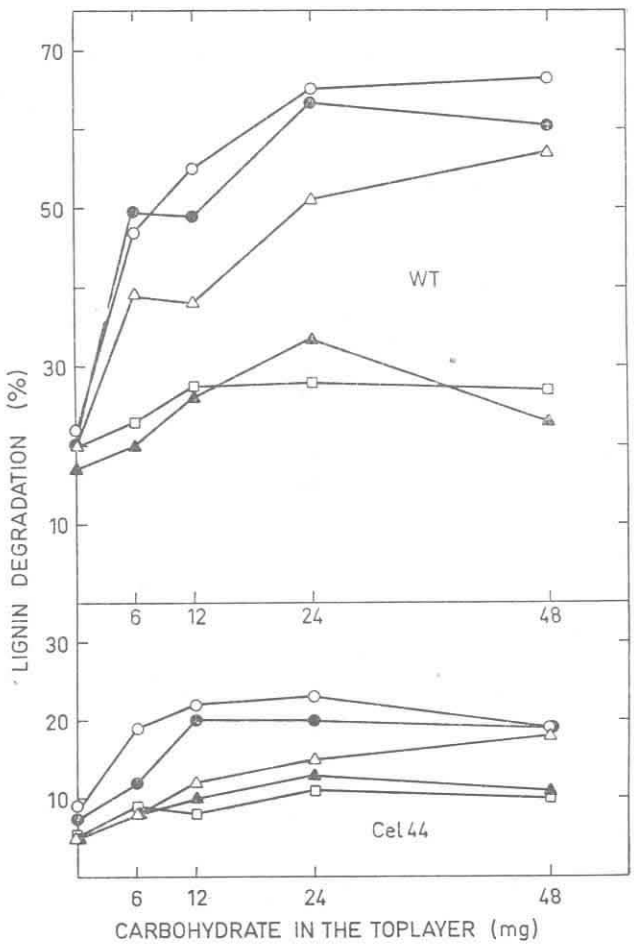

Fig. 2. Degradation of kraft lignin in the presence of different carbohydrates by WT and Cel 44. ○ Cotton linters DP 2000. ● Cotton linters DP 500. △ Walseth cellulose DP 150. ▲ Cellobiose. □ Glucose.

To investigate how cellulose of different DP, cellobiose, and glucose influence lignin degradation by WT and Cel 44, lignin agar plates were prepared containing these additional substrates. *Fig. 2* shows that with WT the degradation of kraft lignin is dependent on the DP of the cellulose. The degradation follows the order cotton DP 2000>cotton DP 500>Walseth cellulose 150> cellobiose or glucose. Fig. 2 shows further that if the amounts of cotton and Walseth cellulose are increased, the lignin degradation increases also, whereas different quantities of cellobiose or glucose do not display this effect. The latter behaviour is probably due to catabolite repression as discussed later.

The cellulase-less mutant Cel 44 shows another degradation pattern. The differences in DP influence the lignin degradation very little. In the presence of cotton

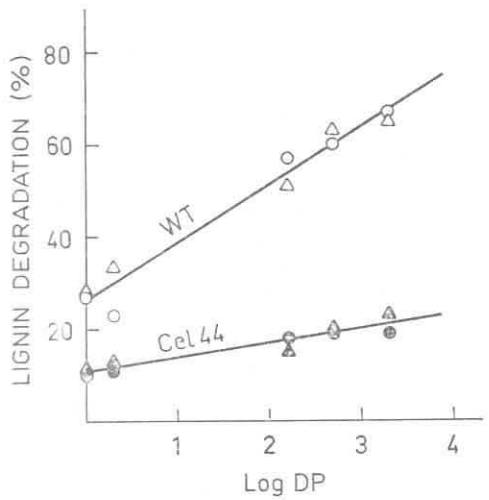

Fig. 3. Degradation of kraft lignin by WT and Cel 44 as a function of the logarithm of the DP of the carbohydrates in fig. 2. ○,● 48 mg and △,▲ 24 mg present of the carbohydrates.

DP 2000 or 500 the degradation of lignin is almost the same at all amounts. The increase in lignin degradation between 0 mg and 12 mg cotton may be due to impurities in the cotton. Walseth cellulose may contain some oligosaccharides which increase the mycelial growth, therefore the lignin degradation. According to *fig. 3* where lignin degradation is plotted as a function of the logarithm of the DP of cotton DP 2000 and 500, Walseth cellulose DP 150, cellobiose (DP 2) and glucose (DP 1), lignin degradation by Cel 44 is almost independent of the DP of the cellulose. The curve for Cel 44 is nearly horizontal, while the curve for WT shows a strong DP dependence.

Xylan and glucomannan influence lignin degradation by WT much less than do cotton or Walseth cellulose *(fig. 4)*. In the presence of 48 mg Walseth cellulose, 56% of the lignin is degraded. The corresponding figure for xylan is 26%, a degradation very similar to that obtained with 48 mg glucose or cellobiose (27% and 23% resp.).

The lignin degradation by Cel 44 in the presence of xylan or glucomannan is nearly the same as that in the presence of Walseth cellulose.

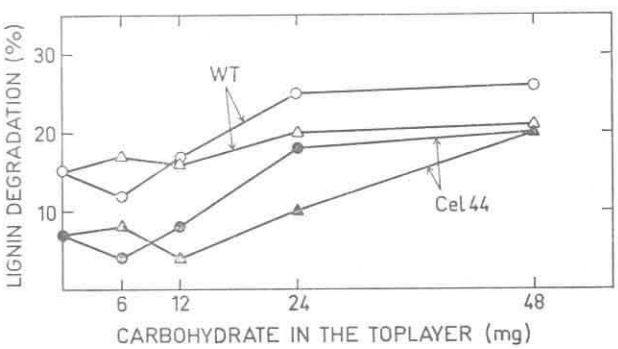

Fig. 4. Degradation of kraft lignin in the presence of xylan and glucomannan by WT and Cel 44. ○,● Xylan. △,▲ Glucomannan.

Coloration and bleaching of kraft lignin — carbohydrate plates by WT and Cel 44

Incubation of WT on lignin-cellulose plates revealed the same colour phenomena on the plates as reported for *Polyporus versicolor* by Westermark and Eriksson (9), who showed that the colour reaction was due to laccase activity. With WT, the colour appeared after about three days and then increased continuously. After about six days the inner region of the brown surface was bleached in some of the plates. The coloration of the plates by WT is indicated in *table 1*. It is obvious from this table and from fig. 2 that both strong coloration, indicating laccase activity, and high lignin degradation are obtained when cotton or Walseth cellulose is present in the plates. With 6, 12 or 24 mg of cellobiose a strong colour appears but the lignin degradation is low. Without carbohydrate, with glucose and with 48 mg cellobiose, little colour develops and little lignin degradation is obtained. The laccase synthesis was totally repressed with 48 mg glucose present.

When WT is growing in the presence of xylan or glucomannan the same colour reactions are obtained as in the presence of up to 24 mg glucose. The lignin degradation is also nearly the same (fig. 2 and 4).

The bleached zones appearing in kraft lignin-cellulose agar plates with *P. versicolor* and reported (9) to be the result of cellobiose dehydrogenase activity, also appeared with wild type *S. pulverulentum*. The extent of bleaching was greatest in the presence of cotton or Walseth cellulose. Little bleaching occurred in the presence of cellobiose, glucose, xylan or glucomannan. The presence of cellobiose in the lignin agar plates thus causes strong coloration but weak bleaching. This shows that the laccase production is normal but that the cellobiose

Table 1. Coloration of kraft lignin by WT on agar media in the presence of different carbohydrates after 6 days. — = no brown coloration; (+) some small brown spots; +, ++, +++, ++++ = diameter of brown coloration 20, 40, 60 and 80 mm respectively.

	Carbohydrate in the top layer (mg)				
Type of carbohydrate	0	6	12	24	48
Cotton DP 2000	+	+++	++++	++++	++++
Cotton DP 500	+	+++	++++	++++	++++
Walseth cellulose DP 150	+	++	+++	++++	++(+)
Cellobiose	+	+++	++++	++++	(+)
Glucose	+	+	+	+	—
Xylan	+	+	+	+	+
Gluco-mannan	+	+	+	+	++

Table 2. Coloration of kraft lignin by Cel 44 on agar media in the presence of different carbohydrates after 6 days. Symbols as in table 1.

	Carbohydrate in the top layer (mg)				
Type of carbohydrate	0	6	12	24	48
Cotton DP 2000	+	++	++	++	++
Cotton DP 500	+	++	++	++	++
Walseth cellulose DP 150	+	+	+	+	+
Cellobiose	+	+	+	(+)	(+)
Glucose	+	+	+	—	—
Xylan	+	+	+	—	—
Glucomannan	+	+	+	—	—

dehydrogenase production is repressed in the presence of high concentration of cellobiose.

The coloration of the lignin-carbohydrate plates by Cel 44 is not as pronounced as for WT. As indicated in *table 2*, the brown coloration for Cel 44 is independent of the amount of carbohydrate and nearly independent of the DP of the carbohydrate.

Some bleaching occurs in the presence of cotton or Walseth cellulose, indicating some production of cellobiose dehydrogenase. The bleaching, however, is much weaker than that caused by WT.

Influence of the moisture content on wood degradation

In the comparison of the degradation obtained on the plates with respect to lignin and carbohydrates in wood, a preliminary study was carried out to determine the influence of the moisture content on the degradation of wood. Vermiculite flasks with wood blocks from birch, pine and spruce respectively and with different amounts of water were incubated with the wild type for four weeks. *Table 3* shows that a high moisture content favours wood degradation by *S. pulverulentum*. The best degradation of birch and pine was obtained at the initial moisture contents of 132% and 138% respectively. The moisture contents in these wood species did not change very much during incubation according to table 3. The best degradation of spruce was obtained when the initial moisture content was 64%. This value increased to 109% after incubation.

Table 4. Analytical data for control blocks of sound wood.

Wood species	Lignin (%)	Glucan[1] (%)	Mannan (%)	Xylan (%)	Other[2] (%)
Birch *(B. verrucosa)*	20.0	40.0	1.5	24.0	14.5
Pine *(P. silvestris)*	30.0	43.5	12.5	5.1	8.9
Spruce *(P. abies)*	28.0	45.8	14.0	5.7	6.5

[1] No correction has been made for glucose released from glucomannan or galacto-glucomannan.
[2] Includes acid-soluble lignin, araban, galactan, extractives, acetyl, uronic acids.

Degradation of wood components by WT and Cel 44

Table 4 shows analytical data for sound wood. It can be seen that the proportion of hemicelluloses is lower in pine and spruce than in birch. Analyses of mannan in birch and of xylan in pine or spruce are not discussed because of the low percentages of these components.

The weight losses and the moisture contents of the wood blocks after 4, 6, 8 and 10 weeks are given in *table 5*. It is obvious that birch wood is easier to degrade than pine or spruce wood by both WT and Cel 44. The moisture content in the birch wood is higher than in the two softwood species. It must be pointed out that the experiments were performed at a temperature 15°C below the growth optimum for *S. pulverulentum* which is 40°C (29).

Table 5 also shows the relative losses of wood components based on the original amounts of components. *Fig. 5* shows the same data for birch. Incubation with WT for a period of 10 weeks gave a total weight loss of 18%, and 12% of the original cellulose was removed. Incubation with Cel 44 for a similar period gave a total weight loss of 11%, but all the cellulose still remained undecayed. During this period, both WT and Cel 44 remove about 30% of the lignin in the wood.

Table 3. Weight losses and moisture contents of birch, pine and spruce sapwood after decay by WT for 4 weeks in the presence of different amounts of water. Initial moisture contents after incubation without fungus for one day are shown within brackets.

Amount of water (ml)	Birch		Pine		Spruce	
	Weight-loss (%)	Moisture content (%)	Weight-loss %)	Moisture content (%)	Weight-loss (%)	Moisture content (%)
20	3.0	38	2.2	32	1.3	31
30	3.5	38	1.8	32	1.3	33
40	6.0	57	2.4	41	1.9	37
50	7.8	96	3.2	41	2.5	43
60	9.0	110 (112)	4.4	89 (74)	4.8	109 (64)
70	10.2	132 (129)	5.1	107 (138)	4.5	147 (115)
80	7.6	131 (120)	4.3	164 (161)	3.0	178 (183)

Table 5. Weight losses[1], moisture contents[2] and relative losses in wood components[3] caused by WT and Cel 44 after different decay times. NT = not tested.

	Incubation period (weeks)	Wildtype						Cel 44					
		Weight loss (%)	Moisture content (%)	Lignin (%)	Glucan[4] (%)	Xylan (%)	Mannan (%)	Weight loss (%)	Moisture content (%)	Lignin (%)	Glucan[4] (%)	Xylan (%)	Mannan (%)
Birch	4	12.1	118	17	7	13	NT	4.6	86	9	0	4	NT
	6	15.7	120	24	10	23	NT	4.5	76	13	—2	15	NT
	8	16.8	122	30	9	34	NT	7.7	110	23	1	35	NT
	10	18.3	125	29	12	22	NT	11.3	123	31	0	32	NT
Pine	4	6.2	42	14	8	NT	22	1.8	57	5	—1	NT	10
	6	6.3	40	9	5	NT	22	2.7	56	8	—3	NT	3
	8	9.7	100	15	7	NT	23	1.5	98	10	—2	NT	2
	10	11.8	88	22	4	NT	18	3.4	84	8	0	NT	14
Spruce	4	8.2	68	8	4	NT	15	0.6	65	2	—5	NT	10
	6	7.0	56	11	5	NT	15	1.5	43	4	—3	NT	21
	8	5.9	55	7	5	NT	25	0.9	61	4	—2	NT	24
	10	8.8	74	15	2	NT	30	1.3	73	5	—3	NT	12

[1] Based on original dry weight
[2] Based on the dry weight of the decayed blocks
[3] Based on original amount of component
[4] Corrected for glucose released from glucomannan and galacto-glucomannan

It is also evident that xylan is degraded simultaneously with the lignin, and that with both WT and Cel 44 the degradation of xylan is much faster than that of cellulose.

When pine is degraded by WT it can be seen (table 5) that mannan is removed slightly faster than lignin which in turn is removed faster than the cellulose. Cel 44 does not remove cellulose and the total weight loss is only about 3%. Lignin and mannan are removed at about the same rate by Cel 44.

In spruce, 30% of the mannan is removed in 10 weeks by the WT. The weight loss is 9%. The same pattern is also shown by Cel 44, although it does not remove glucan at all. Lignin is removed faster than glucan but slower than mannan by both WT and Cel 44.

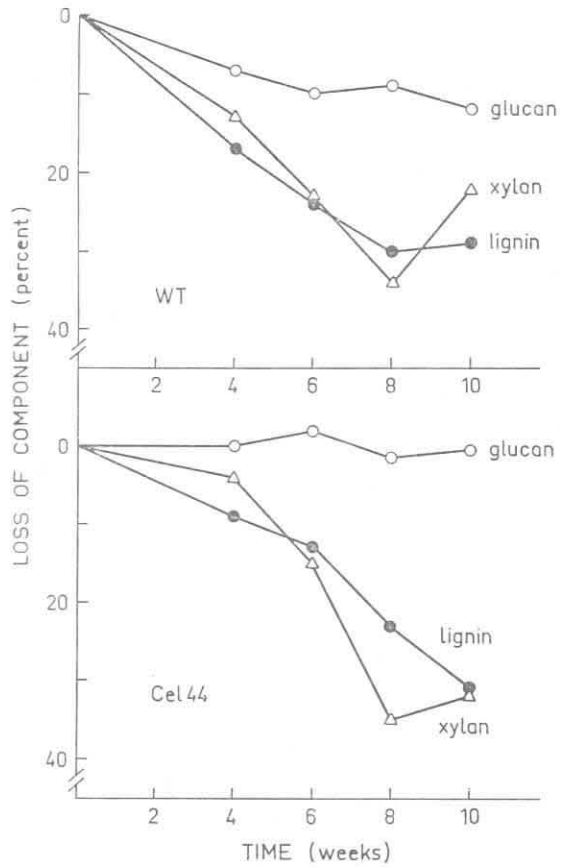

Fig. 5. Degradation of wood components in birch by WT and Cel 44. ○ Glucan. ● Lignin. △ Xylan.

Discussion

It is known from studies of cellulose degradation (30—32) that concentrations of glucose or cellobiose higher than 0.01% cause catabolite repression of cellulase synthesis. Eriksson and Goodell (7) reported that mannanase, xylanase, aryl-β-glucosidase and pectinase production was repressed by glucose. It has also been shown that in addition to glucose also xylose, arabinose, galactose and mannose can repress endo-glucanase production (33). It is thus very common for easily metabolised carbohydrates to act as repressors for the synthesis of hydrolytic enzymes. It seems possible that induction of ligninolytic enzymes is also repressed by glucose, cellobiose or by hemicellulose degradation products. The low degradation of kraft lignin by WT observed in the presence of glucose, cellobiose, xylan or glucomannan (fig. 2 and 4) is probably a result of such a repression.

It is reported here that kraft lignin is strongly degraded by the wild type *S. pulverulentum* when carbohydrates

of high DP are present. It is reasonable to assume that when using cotton linters of a high DP, just enough degradation products are released to allow growth of the fungus but not repression of the ligninolytic enzymes.

It is also shown that lignin degradation by the cellulase-less mutant Cel 44 is very little influenced by the DP of the cellulose present. This is expected, since this mutant produces neither endo- nor exo-cellulases as demonstrated both viscometrically with CMC and in test tubes containing cellulose agar.

Hiroi and Eriksson (8) have shown that the white-rot fungus *Pleurotus ostreatus* can degrade 35% of kraft lignin in silica gel plates in 40 days. In the presence of Walseth cellulose, 60% of the lignin is degraded. Unpublished data from this laboratory also show that other white-rot fungi are able to degrade kraft lignin better in the presence of cellulose. To find fungi without this dependence on cellulose is of course important for the production of biological pulp.

Both laccase and cellobiose dehydrogenase have been connected with lignin degradation (10, 34, 35). Fåhraeus (36) cultivated *Polyporus versicolor* in the presence of 2% glucose. Laccase activity was not detected until the glucose was consumed. This could be due to catabolite represssion and the fact that when starving the fungus started to produce laccase. It has been shown by Froehner and Eriksson (37) that under starving conditions and when protein synthesis is moderately inhibited, *Neurospora crassa* starts to produce laccase. Grabbe et al. (38) have also shown that glucose represses laccase synthesis in *Polyporus versicolor*. When *S. pulverulentum* was cultivated in submerged culture (10) the highest activity of cellobiose dehydrogenase was obtained in the presence of Munktell's Cellulose Powder. Walseth cellulose gave smaller amounts while cellobiose was a poor inducer of cellobiose dehydrogenase.

As mentioned before (see Results), laccase and cellobiose dehydrogenase can be extracted from the coloured and bleached zones, respectively, in kraft lignin agar plates supplemented with cellulose after growth of white-rot fungi (8—10). The coloration and bleaching that appeared in the lignin media in our investigation must be considered to be due to laccase and cellobiose dehydrogenase activity respectively. With WT the strongest coloration and thus the highest laccase activity was obtained on lignin-agar plates in the presence of carbohydrates with a high DP. These plates were also bleached, indicating the action of cellobiose dehydrogenase. Little coloration and bleaching were obtained in the presence of easily metabolized carbohydrates. Strong coloration and bleaching occurring simultaneously were accompanied by extensive lignin degradation.

According to the laccase-test by Käärik (21) and to the coloration of the lignin media reported here, the mutant Cel 44 produces nearly the same amount of laccase as WT. The coloration was not dependent on the DP of the carbohydrate. The cellobiose dehydrogenase production is very small in the mutant and cannot be detected in liquid cultures. In solid lignin agar plates some bleaching appears in the presence of cellulose with high DP. This need not necessarily be the result of reduction of the quinones. It can also be caused by the lignin being metabolized, since kraft lignin can be degraded by Cel 44 also, although to a smaller extent than by WT.

Since bleaching is visible only in connection with coloration, it would be logical to assume that cellobiose dehydrogenase synthesis is coupled with laccase synthesis by a common regulation mechanism. We have shown, however, (35) that a laccase-less mutant of *S. pulverulentum* has strong cellobiose dehydrogenase activity. It thus seems more probable that the induction of cellobiose dehydrogenase is caused by cellulose degradation products. If no cellulose degradation occurs, as with Cel 44, no cellulose degradation products can be formed to induce cellobiose dehydrogenase synthesis. In wild type *S. pulverulentum*, cellobiose induces cellobiose dehydrogenase activity. Cel 44 grows very well on cellobiose as the only source of carbon but no cellobiose dehydrogenase is induced under the conditions used. This may be so because the gene governing the synthesis of cellobiose dehydrogenase is defective in Cel 44. The most likely explanation is, however, that endo- and exo-glucanases and cellobiose dehydrogenase are regulated by a common regulatory gene which has been destroyed in Cel 44, cf. ref. (7).

Since kraft lignin contains a large number of phenolic hydroxyl groups (39) kraft lignin agar is a useful medium when induction of laccase and cellobiose dehydrogenase is studied. However, the best medium for the study of lignin degradation is wood. The best growth of wild type *S. pulverulentum* on wood was obtained when the initial moisture content was 64 to 139%. This result is in good agreement with results obtained by Henningsson (40). He reported that the maximum rate of decay by birch-attacking fungi after three months was obtained when the initial moisture content ranged from 60 to 120%.

S. pulverulentum degrades birch wood easier than pine or spruce wood. The high content of xylan in birch may be one reason for this. Preliminary experiments (10) have indicated that *S. pulverulentum* produces an enzyme xylobiose:quinone oxidoreductase which oxidizes xylobiose in the same way as cellobiose dehydrogenase oxidizes cellobiose. The fact that hardwood is easier to degrade than softwood has also been reported by Kirk and Moore (5) and by Bergman and Nilsson (29). It seems that water can penetrate hardwood more easily which results in a more favourable environment for the fungus and its enzymes. Different amounts and types of lignin may also influence the rate of degradation (2). Wild type *S. pulverulentum* removes xylan in birch wood faster than it removes cellulose but lignin is degraded simultaneously with xylan. This supports the theory (41) that hemicellulose and lignin are intimately associated. Kirk and Moore (5) investigated decay of birch wood *(Betula alleghaniensis)* by several white-rot

fungi but only one—*Fomes ulmarius*— removed xylan faster than cellulose.

The cellulase-less mutant Cel 44 did not remove cellulose from birch wood, but in spite of that there was a weight loss of 11% after 10 weeks and 31% and 32% respectively of the lignin and xylan were removed. Cel 44 could not clear xylan agar in test tubes but in wood, xylanase is obviously induced.

F. ulmarius, mentioned above, showed the same degradation pattern as Cel 44. At a weight loss of 15%, only 3% of the cellulose was removed by *F. ulmarius*. Lignin was removed slightly faster than xylan. Henningsson et al. (42) reported that when the Basidiomycete P-Bl belonging to the *Peniophora cremea*-group caused a weight loss of 15% in birch, 50% of the lignin was degraded but only 2% of the cellulose. However, as the decay proceeded the fungus started to attack the cellulose also. Hemicelluloses were degraded faster than cellulose. The hypothesis put forward by Kirk and Moore (5) that lignin cannot be removed unless either xylan or cellulose is removed at the same time thus still seems to be correct.

Mannan in pine or spruce sapwood is removed faster than cellulose by wild type, *S. pulverulentum*, while lignin is removed somewhat faster than cellulose but slower than mannan. This is in agreement with the assumption by Kirk and Highley (43) that hemicellulose in conifers is the most rapidly removed component. The mutant Cel 44 degrades lignin in both pine and spruce but cellulose is not degraded.

This report shows that it is possible to obtain a mutant of a white-rot fungus which is able to degrade lignin in wood without degrading the cellulose and without having any or at least very little cellobiose dehydrogenase production.

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